Blocking HMGB1 signal pathway protects early radiation-induced lung injury

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Abstract: It has been reported that HMGB1 participated in various types of lung injury. In this study, we explored whether blocking HMGB1 has a preventive effect on the early radiation-induced lung injury and investigate the mechanism. Mice model of radiation-induced lung injury were accomplished by a single dose irradiation (15 Gy) to the whole thorax. Irradiated mice were treated with HMGB1-neutralizing antibody intraperitoneally dosed 10 μg, 50 μg, 100 μg/mouse respectively and were sacrificed after one week post-irradiation. Lung tissue slices were stained by H&E, and alveolitis was quantified by Szapiel scoring system. The level of cytokines TNF-γ in bronchoalveolar lavage fluid was detected by ELISA method. And p65NF-κB, p50NF-κB protein expression in mice lung tissues was detected by Western blot analysis. The results showed that blocking HMGB1 inhibited the inflammatory response, and thereby decreased the degree of alveolitis of irradiated lung tissue. In addition, HMGB1 antagonist can restrain the expression of type Th2 or Th17 derived inflammatory cytokines TNF-α, IL-6 and IL-17A, promote the expression of Th1 type cytokines INF-γ, and inhibit p65 NF-κB but promote p50 NF-κB activation, which promoted the resolution of the radiation-induced inflammatory response. In conclusion, blocking HMGB1 can reduce the degree of early radiation-induced lung injury, and its mechanism may be related to the promotion of p50NF-κB activation and its downstream molecules expression. Inhibiting HMGB1 may be a new target to deal with early radiation-induced lung injury.

Keywords: HMGB1, radiation-induced lung injury, NF-κB

Introduction

Radiation-induced lung injury (abbreviated as RILI) is a common complication of thoracic tumor radiotherapy, which is also one of important factors of limiting target dose and then influencing the local control rate. Studies have found that the failure of resolving early or acute pulmonary inflammation induced by radiation, would lead to the lung damage accumulated, and result in the progression towards chronic inflammation or fibrosis eventually [1]. Therefore, if the degree of inflammation can be controlled in the early stage of injury, lung tissues would be protected from deterioration. In previous studies, by blocking a single inflammatory cytokine such as TNF-α, IL-17A, TGF-β, etc., radiation-induced lung injury can be alleviated to some extent [2-4]. But the effect is not always too good. Therefore, it suggests that there may be other more important cytokines involved in the process of radiation-induced lung injury.

High mobility group box protein 1 (abbreviated as HMGB1) is an important mediator of inflammation found in recent years, which can promote inflammatory cell activation and pro-inflammatory cytokine production and secretion [5, 6], enhance adhesion of monocytes and promote p50 NF-κB activation, which promoted the resolution of the radiation-induced inflammatory response. In conclusion, blocking HMGB1 can reduce the degree of early radiation-induced lung injury, and its mechanism may be related to the promotion of p50NF-κB activation and its downstream molecules expression. Inhibiting HMGB1 may be a new target to deal with early radiation-induced lung injury.
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valid target and a new strategy in prevention and treatment of RILI. As we know, NF-κB is an important transcription factors in the downstream of HMGB1 signaling pathway [12]. Studies have shown that, p65 NF-κB (p65) and p50 NF-κB (p50), the two important NF-κB sub-units, which were involved in the resolution and progression of pulmonary inflammation by mediated different inflammatory cytokines [13].

In this study, HMGB1 neutralizing antibodies were used to treat mice in order to explore whether blocking HMGB1 has a preventive effect on the early radiation-induced lung injury and investigate the mechanism.

Materials and methods

Animal and reagents

Male C57BL/6J mice (SPF grade, 8 weeks old, 21±2 g in weight) were purchased from Vital River Laboratory Animal Technology (Beijing, China). Rabbit anti-mouse HMGB1 neutralizing antibodies and rabbit IgG2a, were purchased from R&D Systems (Minneapolis, MN, US); mouse anti-phospho-p65/50, p65/50, COX-2 and β-actin Abs available from Cell Signaling Technology (Danvers, MA, US); ELISA kits for mouse TNF-α, IL-17A, IL-6, INF-γ cytokine were purchased from eBioscience (San Diego, CA, US); Protein extraction kits for western blot assay were purchased from Qiagen (Toronto, ON, Canada). H&E staining kits were obtained from Beyotime biotechnology Research Institute.

Animal model preparation and intervention

The study protocols were approved by the Ethics Committee of Liaocheng People’s Hospital. Animal models for RILI were accomplished according to the method described in our previous published papers [14]. In brief, a plexiglas jig was designed for fixed mice, which contained 12 mice at the same time. The whole thorax of mice was irradiated by ELEKTA precise linear accelerator at a single dose of 15 Gy only once [15]. The parameters of the radiotherapy plan were as follows: beam energy: 6MV-photons; dose-rate: 3.0 Gy/minute; source surface distance (SSD): 1 m; radiation field (FS): 40 cm×1.8 cm.

The 60 mice were randomly divided into the following groups: Sham, RT (irradiation group, n=10), RT+Ab1 (irradiation plus 10 μg anti-HMGB1 Ab, n=10), RT+Ab2 (irradiation plus 50 μg Ab, n=10) and RT+Ab3 group (irradiation plus 100 μg Ab, n=10). Anti-HMGB1 antibodies were injected 10 μg, 50 μg and 100 μg intraperitoneally for the later three groups. RT+IgG group was injected with the same volume of 100 μg non-specific IgG.

Specimen preparation

After one week post-irradiation, the mice were sacrificed and the lavage fluid and lung tissue were collected immediately. Left upper lobe lung tissue was fixed in 10% formalin for histomorphological analysis. Right lung tissue was stored at -80°C for protein extraction.

BALF were obtained according to the method of reference [16]. Briefly, after the mice were fully exposed trachea, sterile catheter is inserted and secured. After instilling 0.5 ml saline twice, bilateral bronchoalveolar lavage fluid was collected. BALF 0.9-1.0 ml was acquired per mouse by this method. BALF supernatant was frozen at -20°C refrigerator for ELISA analysis.

Histopathological detection and analysis

The lung tissue were fixed in 10% formalin overnight, embedded in paraffin after dehydration, and sliced to a thickness of 3 μm. Slices were stained by H&E, and then alveolitis were observed by light microscopy. Lung tissue inflammation were evaluated and quantified (0-III level) according to the method established by Szapiel [17].

ELISA for cytokines in BALF

The contents of HMGB1, TNF-α, IL-17A, IL-6, and INF-γ in BALF were detected by ELISA using kits according to the manufacturer’s instructions strictly.

Western blotting for protein NF-κB and COX-2

Proteins were extracted from lung tissue stored in -80°C refrigerator using a Qproteome plasma nuclei protein kit. Total protein concentrations were determined using Bradford kit. SDS-PAGE and Western blotting were carried out in accordance with specification.

Statistical analysis

Data are presented as mean ± standard deviation (SD). One-way ANOVA was used to compare
the significance of numerical data between groups. Comparison of categorical data between two groups was performed with chi-square test. Values were considered significantly different when \( P<0.05 \). All analyses were carried out by SPSS 19.0 software.
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In this study, we detected the levels of some inflammatory cytokines (TNF-α, IL-17A, IL-6, INF-γ) related closely with radiation-induced lung injury in bronchoalveolar lavage fluid. Among these cytokines, TNF-α, IL-17A, IL-6 are elevated in irradiated mice, while INF-γ was reduced. Compared with irradiation group (RT group), blocking HMGB1 can reduce inflammatory cell infiltration (Figure 1E, 1F), reduce alveolar lavage cytokines TNF-α, IL-17A, IL-6 expression (Figure 3), and improve the content of INF-γ factor (Figure 4). That is to say, HMGB1 antagonism suppressed the recruitment and infiltration of inflammatory cells as well as the expression of the inflammatory cytokines TNF-α, IL-17A, and IL-6 in the irradiated mice.

Blocking HMGB1 promoted P50 and its downstream signal activation

To explore the mechanism of HMGB1 antagonists modulating inflammation, we further examined NF-κB activation and its downstream molecules expression after HMGB1 blockade (100 μg antibody group). The results show that, compared with RT group, blocking HMGB1 can reduce p65 activation and further promote the activation of P50, elevate the ratio of the activation of P50 and p65 (Figure 5A, 5B), and also increased the downstream expression of COX-2 which initiated the signal of subsided inflamma-
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The above results suggest that the mechanism of alleviated alveolitis produced by blocking HMGB1 signal may be related with the promotion of p50NF-kappa B activation.

Discussion

HMGB1 is an inflammatory-related protein secreted by activated monocytes and macrophages, which is also a nuclear protein. Studies have found that the inflammatory response of HMGB1 gene-/- necrotic cells is significantly milder than that of the HMGB1 wild-type cells [18]. Recombinant HMGB1 may cause liver tissue, lung tissue, joints and other injuries and diseases [10, 19-27]. Moreover, anti-HMGB1 neutralizing antibodies play protective roles in experimental animals against sepsis, arthritis, ischemia-reperfusion injury, BLM-induced lung injury and lung graft rejection in pathological symptoms, tissue lesions, mortality, etc. These studies have confirmed the HMGB1 plays an important role in a variety of inflammatory diseases. So, we believe that the blockade of HMGB1 might attenuate or prevent the progression of the disease, and the effective inhibition of HMGB1 release may play an active role in preventing inflammatory progression.

We have confirmed in previous research, HMGB1 gene and protein expression were elevated significantly in early radiation pulmonary injury in lung tissue of irradiated mice. In this study, we found that HMGB1 expression was also increased in BALF, which indicated that HMGB1 is involved in the early inflammatory process of radiation-induced lung injury. Histopathological results showed, anti-HMGB1 antibody treatment (group 50 μg and group 100 μg) markedly reduced the expression of COX-2 in the downstream of NF-κB signal pathway. The phosphorylation of transcription factors was detected by Western blotting with the specific anti-phospho-Abs. Data are representative immune blots of three independent assays. Data are presented as the mean ± SE (n=3/group).

[Figure 5. A, B: Neutralization of HMGB1 (100μg anti-HMGB1 antibody) markedly attenuated the radiation-induced activation of transcription factors NF-kB. C, D: Neutralization of HMGB1 (100 μg anti-HMGB1 antibody) markedly reduced the expression of COX-2 in the downstream of NF-κB signal pathway. The phosphorylation of transcription factors was detected by Western blotting with the specific anti-phospho-Abs. Data are representative immune blots of three independent assays. Data are presented as the mean ± SE (n=3/group).]
is related closely with RILI were elevated in BALF of irradiated mice. After anti-HMGB1 antibody treated, TNF-α, IL-6 and IL-17 expression were significantly decreased. And the effect of dose 100 μg and 50 μg is obviously better than that of 10 μg dose group, which also shows a dose dependent manner. Western blot detection showed that, blocking HMGB1 can reduce p65 activation and further promote the activation of p50, and the increase of the ratio of the activation of p50 and p65 (Figure 5A, 5B).

Wynn et al [28] proposed that the nature of the immune microenvironment determines the outcome of tissue inflammation. Th1-type dominant immune microenvironment suppresses the development of tissue inflammation and fibrosis, while Th2, Treg type dominant promote. The study found that blocking HMGB1 can inhibit Th2-type cytokines TNF-a, IL-6, IL-17A and promote Th1-type cytokine IFN-γ expression, which suggests that blocking HMGB1 could shift the Th2-polarized response toward a Th1-polarized response, and eventually lead the inflammation to subside or reduced.

NF-κB is an important transcription factor in the development of inflammation. It contains subunits p65 and p50, which can form p65-p50 heterodimer and p50-p50 homodimer to transcript different inflammatory factor expression respectively, and mediate tissue injury and inflammation subsided [13]. We found that the two subunits can significantly be activated after thoracic irradiation in mice lung tissues. After application of anti-HMGB1 antibody, the levels of p65 activation were significantly lower, p50 activation was significantly enhanced, and the ratio of activated p50 and p65 was significantly increased, which suggested that p50-p50 homodimer gradually replace p65-p50 heterodimer involved in radiation pneumonitis [29]. p50-p50 homodimer is considered as an important nuclear transcription factor which plays key roles during the initial stages of the inflammatory process including transcription of COX-2 and other inflammatory protein [30].

This study shows that blocking HMGB1 can prevent early radiation pneumonitis from deterioration by restoring local inflammation outcome of lung injury. And blocking HMGB1 can induce local immune microenvironment towards Th1 polarization contributed to resolution of inflammation. In the local lesions, inflammation resolution and inflammatory response exist simultaneously. In fact, inflammation resolution also persists even in the context of chronic inflammation, but the active process is suppressed or weakened [29]. Blocking HMGB1 can help the body to restore and strengthen the effects. These data indicate that targeting HMGB1 can attenuate early radiation related inflammation by promoting the resolution of the inflammatory response. Thus, HMGB1 antagonism showed considerable efficacy against radiation-induced acute pulmonary inflammation.

In summary, we believe that HMGB1 as a potential therapeutic target on radiation-induced lung injury has important significance in clinical research and drug development.

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Disclosure of conflict of interest

None.

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